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Expression of lactoferrin in the kidney: Implications for innate immunity and iron metabolism

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Expression of lactoferrin in the kidney: Implications for innate immunity and iron metabolism.

Background. Sequestering of free iron by lactoferrin (LF) is important in the defense against bacteria. In a screening for LF expression in various organs, high levels of LF mRNA were detected in human kidney. This indicated that LF is produced by the kidney and that it may participate in innate immunity of this organ.

Methods and Results. Antibody staining and in situ hybridization of paraffin-embedded kidney sections showed that LF is expressed in cells lining the distal collecting ducts of the medulla. High levels of both protein and mRNA were detected in these cells. However, a clear difference in the distribution of mRNA and protein within the tissue was observed. LF mRNA was detected along a relatively large portion of the tubuli, whereas LF antigen was found mainly in the very distal regions of the same tubuli. This indicates that LF is released by large regions of the tubuli and possibly reabsorbed in the most distal parts. Using enzyme-linked immunosorbent assay, only very low LF levels were detected in urine.

Conclusion. The present study shows that LF is produced by the kidney and that both LF mRNA and protein are distributed in a highly ordered fashion. This latter finding, together with the very low levels of LF detected in urine, indicates that LF may contribute to the immune defense in the kidney by reduction of available free iron in the urine. Other possibilities are that LF may play a role in the iron metabolism by recovering free iron from urine and making it available for metabolic use, and that LF may participate in the antioxidant defense systems protecting the kidney against nonmicrobial oxidative injury, that is, ischemia, reperfusion and inflammation.

Lactoferrin (LF) is an iron-binding protein that is related in structure to transferrin. LF was first isolated from human breast milk [1] and was found later in several other organs, such as kidney, gallbladder, lung, pancreas, prostate, seminal vesicle, gut, and liver [2–7]. Specialized cells in these organs seem to be responsible for the production of LF, and several cell lines originating from these organs have been shown to synthesize LF [7]. In addition, LF is particularly abundant in secretions, such as tears, saliva, seminal plasma, and vaginal mucus [8–11]. LF is also one of the main immune proteins of the neutrophil granulocytes. In bone marrow, the level of LF mRNA is high but ceases after lineage commitment, and no LF mRNA is found in monocytes and mature neutrophils [polymorphonuclear cells (PMNs)] [12, 13]. Most cell lines derived from hematopoietic cells also seem to have lost the capability of synthesizing LF [7]. In PMNs, the protein is stored in special (secondary) granules where it resides until cellular activation [14]. These cells degranulate upon contact with a foreign antigen, via a receptor-mediated mechanism, and release a number of antibacterial and antiviral peptides, including LF [reviewed in 15]. This process is the major source of LF in plasma. After degranulation, LF is cleared from the circulation by several separate mechanisms mainly involving cells of the macrophage/monocyte cell lineage [16, 17]. These cells have specific high-affinity receptors that internalize LF and transfer the bound iron to ferritin, accompanied by the destruction of LF [18].

The major function of LF probably resides in its bactericidal effects, either by sequestering free iron [reviewed in 19] or by the effects of lactoferricin, an antibacterial peptide generated by proteolytic cleavage of LF [20]. In addition, LF has been shown to function as a transcription factor [21].

Lactoferrin has previously been shown to be present in urine and fecal samples. However, the levels in these samples are relatively low. In infant urine, the maternal milk has been implicated as the source of LF [22], whereas the levels in fecal samples of adults probably reflect secretion from the gallbladder and pancreas [23]. Cells in the rectal mucosa have also been shown to produce LF [23, 24]. The production of LF by pancreas and by the rectal mucosa may be of importance for the control of the microbial flora of the duodenum [3].

Key words: urine, lactoferricin, antibacterial, immune defense, free iron.

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Several reports have previously indicated the presence of LF in kidney [2, 25]. However, previously it has not been known whether LF actually is expressed by resident kidney cells or whether the protein originates from infiltrating neutrophils. In an earlier report, to our surprise we detected mRNA for LF in kidney [26]. In the present study, we investigated the source and the distribution of kidney LF, both at the mRNA and protein levels. We show that LF is expressed by cells lining the distal tubules of the medulla. Furthermore, our results indicate that LF is produced and released in the upper region of the tubuli and reabsorbed further downstream along the tubuli, most likely after binding free iron.

METHODS

RNA preparations

Total RNA was prepared from tissue samples from seven different human kidneys. Four of these kidneys (samples 4 through 7) were transplanted and removed because of rejection [one acute vascular and three kidneys with chronic vascular rejection (CVR)]. The three remaining kidneys (samples 1 through 3) were removed because of the presence of small tumors (hypernephromas). Normal liver tissue was obtained from areas present around the tissues obtained by resection of a benign tumor. Approximately 25 μg of total RNA from each tissue sample were separated by electrophoresis on denaturing agarose gels containing formaldehyde, followed by transfer to Hybond N+ nylon membranes (Amersham, Buckinghamshire, UK). The Northern blots were hybridized with a ^{32}P-labeled LF cDNA fragment in a solution containing 7% sodium dodecyl sulfate (SDS) + 0.25 mol/L Na_2HPO_4. The hybridization was carried out at 65°C overnight and washed under high-stringency conditions (1% SDS + 0.02 mol/L Na_2HPO_4). The filter was stripped and used for rehybridization with the β-actin cDNA as probe.

Polymerase chain reaction amplification and nucleotide sequence analysis

Lactoferrin is homologous to other transferrin-like gene family members. To study the origin of the hybridization signals observed during Northern blot analysis and in situ hybridizations, polymerase chain reaction (PCR) primers were designed to amplify both transferrin and LF mRNA. The primers were directed against regions that showed full or almost full sequence identity between LF and transferrin. In the region covered by one of the primers, LF and transferrin differed in sequence in two positions. In these positions, both nucleotides were added during synthesis to give a mixture of two sequence specific primers in equal amounts (mixed synthesis). The sequence of the primers is as follows: 5′ primer, 5′-TGGC CCRGGTCCCTTCTCATRC-3′, and 3′ primer, 5′-TGG CAGGACTTCTTGCCTTTCA-3′.

The primers were used in several amplification reactions, and the nucleotide sequence of the PCR products was determined. The sequence was shown to be identical to the LF sequence (data not shown). However, minor background bands were detected, which may indicate low levels of transferrin mRNA in the kidney. However, the levels were below 10% of the LF signal.

Immunohistochemistry

A standard protocol for immunostaining using the peroxidase antiperoxidase (PAP) amplification system was used. Briefly, 4 μm sections were cut from paraffin-embedded, formalin-fixed kidney tissues. The sections were deparaffinized and rehydrated. A rabbit polyclonal anti-LF antibody (a kind gift from Professor Inge Olsson, Lund, Sweden) was used at a dilution of 1:200 as primary antibody, followed by application of a sheep antirabbit antibody diluted 1:30 and finally a PAP-complex (Dako, Carpinteria, CA, USA) diluted 1:80. 3-Amino-9-ethyl carbazole was used to visualize the PAP complex. Endogenous peroxidase was blocked by H_2O_2 before application of the first layer. The rabbit polyclonal anti-LF antibody was tested for reactivity against purified transferrin and LF in an Ouchterlony assay. The antisera showed precipitin lines only against purified LF (data not shown). No cross-reactivity was detected against transferrin (data not shown).

Enzyme-linked immunosorbent assay

A LF-*f*-EIA kit (BIOXYTECH.S.A.) was purchased from R&D Systems (Minneapolis, MN, USA) and was used according to the manufacturer's protocol. Samples were collected, prepared, and stored until being assayed. Briefly, the urine samples were centrifuged for five minutes, and 1.5 mL of the supernatants were stored at −20°C. Tear samples were collected and kept in −20°C. Samples of saliva were first sonicated and then kept at 4°C. Blood samples (10 mL) were withdrawn and transferred either to nonclotting glass tubes [containing ethylenediaminetetraacetic acid (EDTA)] or to Vacutainer tubes for rapid coagulation. The samples of EDTA-treated blood were diluted 1:1 with EDTA-phosphate-buffered saline (PBS) and further fractionated into plasma, peripheral blood leukocytes (PBLs), and PMNs by density gradient centrifugation using Polymorphprep, Nycomed AS. Purified cells were washed three times in PBS, resuspended in 1 mL, and counted. From PBLs and PMNs, triplicates of 50,000 cells were prepared on cytospin glass slides, followed by Giemsa staining to determine the purity of the cell populations. NP-40 was used to lyze PBL and PMN before the enzyme-linked immunosorbent assay (ELISA) test.

In situ hybridization

Four micrometer sections from paraffin-embedded kidney samples were used in in situ hybridization studies to localize LF-mRNA. A DNA fragment spanning the 3′-prime end of the LF cDNA was used for the ^{35}S radio labeling of the antisense (by using the T7 polymerase) and the sense probes (by using the Sp6 polymerase). In situ hybridization was performed as previously described [27]. Briefly, the sections were dewaxed in Xylen two times for five minutes each followed by washing in ethanol. Next, the sections were rinsed briefly in water followed by PBS. Protease treatment was performed in a humidified chamber using proteinase K at a concentration of 100 μg/mL in a solution of 0.1 mol/L Tris-HCl and 5 mmol/L EDTA for 15 minutes at 37°C. Sections were then washed in PBS and acetylated by incubation in 0.1 mmol/L triethanolamine, pH 8.0, and 0.25% (vol/vol) acetic anhydride for 10 minutes at room temperature. Approximately 5×10^7 cpm/mL of ^{35}S-labeled RNA probe were applied onto the sections in a solution containing 2 × standard saline citrate (SSC), 50% formamide, 10% (wt/vol) dextran, 1 mg/mL of tRNA, 1 mg/mL of salmon sperm DNA, 2 mg/mL of bovine serum albumin, and 100 mmol/L dithiothreitol (DTT) and were hybridized in a humidified chamber for three to four hours at 50°C. After hybridization, the sections were washed and treated with RNase A and T1. After several washings, the sections were dehydrated and subjected to autoradiography with photographic emulsion NTB2 (Eastman Kodak, Rochester, NY, USA). The sections were then exposed for two weeks at 4°C. Slides were developed and counterstained with hematoxylin and examined for LF staining and photographed using a Canon or Leitz light microscope.

RESULTS

Northern blot analyses

To determine whether the levels of LF mRNA differ between different regions of the kidney and whether the condition, that is, the presence of tumors, transplantation rejection, or infection, of the kidney influences the LF mRNA levels, samples from seven different kidneys were studied by Northern blot analysis. Four of these kidneys (samples 4 through 7) were transplanted and removed because of rejection (one acute vascular and three kidneys with CVR). The three remaining kidneys (samples 1 through 3) were removed because of the presence of small tumors (hypernephromas). The tissues saved as normal were macroscopically and microscopically normal tissue from samples, which were removed because of the presence of small tumors. Although a large variation in expression levels was observed, all samples were found to contain LF mRNA (Fig. 1). The size of the LF mRNA was approximately 2.5 kb, which is in agreement with the size reported for LF mRNA from other organs [7]. Large variations in expression levels were observed for different regions within the kidney. High levels were observed in samples originating from the medulla, whereas low levels were found in samples from the cortex. There were no obvious differences between samples originating from normal kidneys, rejected kidneys, or kidneys with small tumors (Fig. 1).

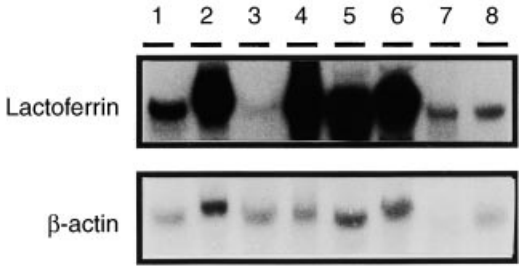


Fig. 1. Northern blot analysis of eight different human tissue samples: three normal (lane 1 through 3), one acute vascular rejected (lane 4), three chronic vascular rejected kidneys (lane 5 through 7), and one normal liver (lane 8). A large variation in expression levels is observed between different patients. However, a good correlation was observed between the amount of cortex or medulla and the expression levels in each sample. Lanes 1, 2, 4, 5, and 6 contain tissue from both medulla and cortex. In contrast, lanes 3 and 7 contain almost only cortical tissue. A low level of lactoferrin (LF) mRNA was also detected in the liver sample. β-actin was used as a control to normalize the loading of RNA in each sample.

Lactoferrin is homologous to other transferrin-like gene family members. A slight possibility thereby exists that the hybridization signals observed in the kidney samples do not originate from LF but instead are from cross-hybridization to transferrin mRNA. The probe used during the Northern blot analysis and the in situ hybridization originate from the 3′ noncoding region of the LF mRNA, a region that shows a low degree of sequence identity between LF and transferrin mRNA. The risk of cross-hybridization is thus very low. However, we still wanted to confirm the identity of the hybridization signals. A PCR-based analysis of the mRNA was therefore performed. PCR primers, which amplify both transferrin and LF mRNA with equal efficiency, were used during the amplification reaction, and the nucleotide sequence of the PCR products was determined. The nucleotide sequences were found to be identical to the LF sequence, and only minor background bands were detected (data not shown). In the kidney, LF is the predominately expressed member of the transferrin-like gene family.

Immunohistochemistry

To study the presence of LF antigen in different regions of the kidney, sections from different parts of the

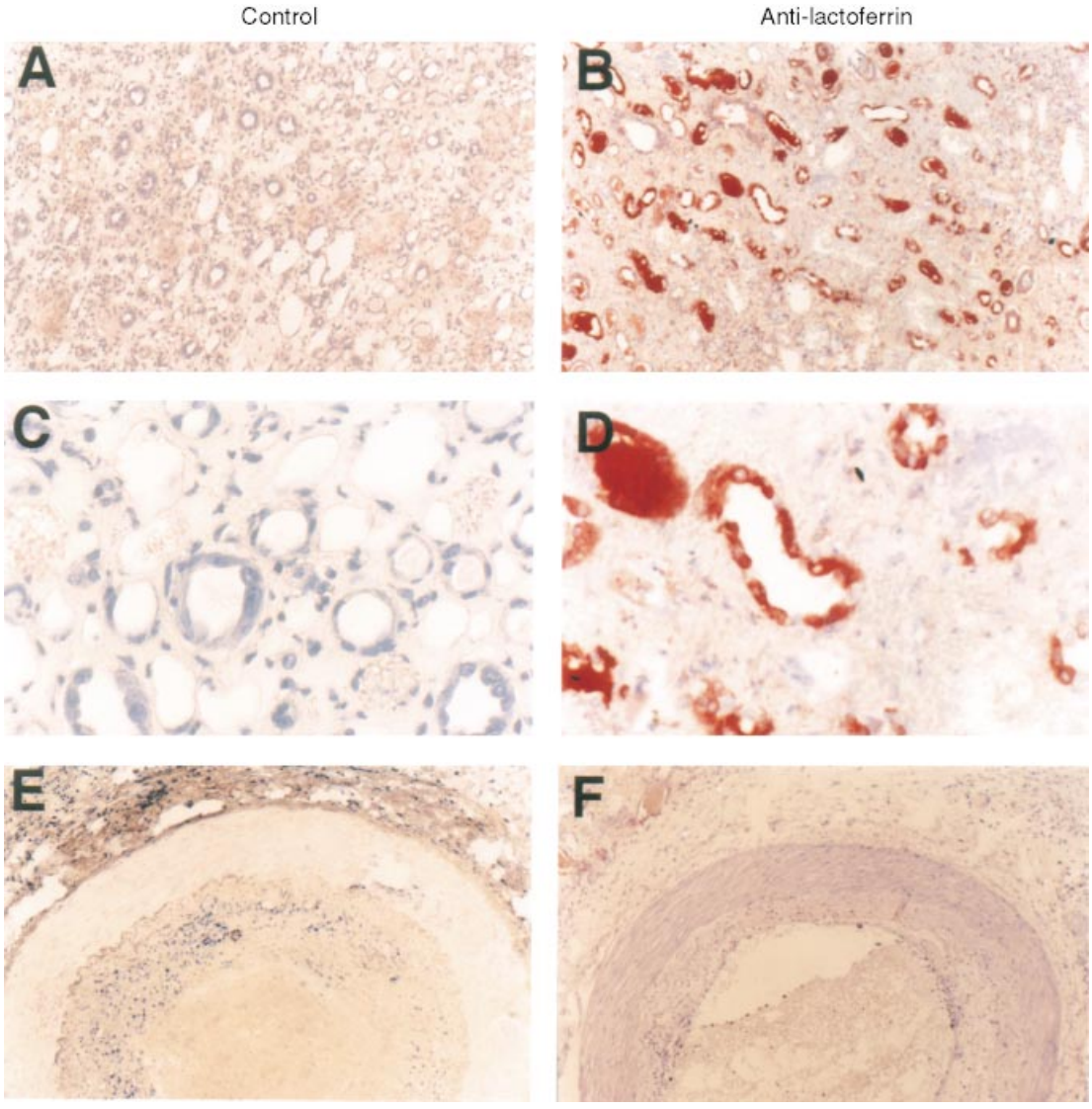


Fig. 2. Immunohistochemical localization of lactoferrin (LF) in sections of a normal human kidney. Sections of the kidney were incubated with a polyclonal rabbit anti-LF antibody (*B, D*, and *F*) or with a normal rabbit serum as control (*A, C*, and *E*). (B and D) These show regions from the medulla where strong staining of distal collecting tubules is observed. In contrast, no staining was detected in tissue sections originating from the cortical region of the kidney (data not shown). All blood vessels were negative (F).

kidneys were stained with an antibody against LF. Staining for LF was found in tubular structures of the medulla (Fig. 2 B, D), but no signal was detected in the cortical region (data not shown). Based on morphological examinations, the structures that showed positive staining were identified as the distal collecting tubules (Fig. 2 B, D). All samples examined, 1 through 3 and 5 through 7, were positive for LF (data not shown). However, not all tubules stained positively, indicating that LF expression is restricted to certain tubuli or to certain regions of the tubuli. No staining was detected in or around blood vessels (Fig. 2F), and no difference between normal and rejected kidneys was observed (data not shown). Furthermore, we could not see any staining of tumor tissues present in two of the otherwise normal kidneys, indicating that there is no correlation between LF expression and the state of transformation (data not shown).

Enzyme-linked immunosorbent assay

How much of the LF produced in the kidney appears in the urine, and what is the role of LF in the kidney? To address these questions, we decided to determine the LF levels in urine and to relate these values to LF levels in other human organs and secretions. Urine, tears, saliva, and blood were collected from six individuals and were prepared as described in the **Methods** section. The LF levels in these samples were determined by a sandwich ELISA (Table 1). Low levels of LF were detected

Table 1. Lactoferrin levels in urine, saliva, tears, plasma, serum, PBL, PMN. The levels were determined with a commercial ELISA kit (lactoferrin-*f*-EIA kit (BIOXYTECH.S.A.).

Samples	*N**	Lactoferrin levels	Range
Urine	6	75 ng/mL	14–145
Saliva	6	51 μg/mL	22–72
Tears	5	2.2 mg/mL	1.5–3.3
Plasma	4	1.0 μg/mL	0.8–1.3
Serum	4	2.8 μg/mL	1.5–4.3
PBL	4	0.4 μg/10 × 7 cells	
PMN	4	3.0 μg/10 × 7 cells	

Abbreviations are: PBL, peripheral blood leukocyte; PMN, polymorphonuclear neutrophil.

* *N* indicates the number of tested individuals.

in urine (average concentration 75 ng/mL). Intermediate levels were found in serum and plasma. High levels were found in saliva and tears. The concentration within individual blood cell populations was also determined. Blood cells was subdivided into PMN, PBL, plasma, and serum. To determine the purity of the cells in these samples, the PMN and PBL cells were fixated on cytospin glass slides following counterstaining with hematoxylin. Approximately 40 fg/cell was detected for total PBL and 0.3 pg/cell for purified PMNs (neutrophils). Approximately 10% neutrophils were detected on the slides with total PBL (data not shown). This observation may explain why the PBL samples also were positive in the ELISA.

In situ hybridization

To further study the synthesis of LF in the kidney, we analyzed kidney samples by in situ hybridization. A cDNA fragment spanning the 3′ end of LF mRNA was used as the probe. Both sense and antisense probes were employed to study the same kidney samples as those used for the immunohistochemical analysis. Using the antisense probe, strong staining was seen in the collecting tubules of the medulla (Fig. 3 B, D, and F). In contrast, no expression was observed in the cortical region, in blood vessels, or in other regions that may contain infiltrating neutrophils (data not shown). In addition, no hybridization was detected in glomeruli (data not shown). Using the sense probe, no staining was detected in any region of the kidney (Fig. 3 A, C, and E).

DISCUSSION

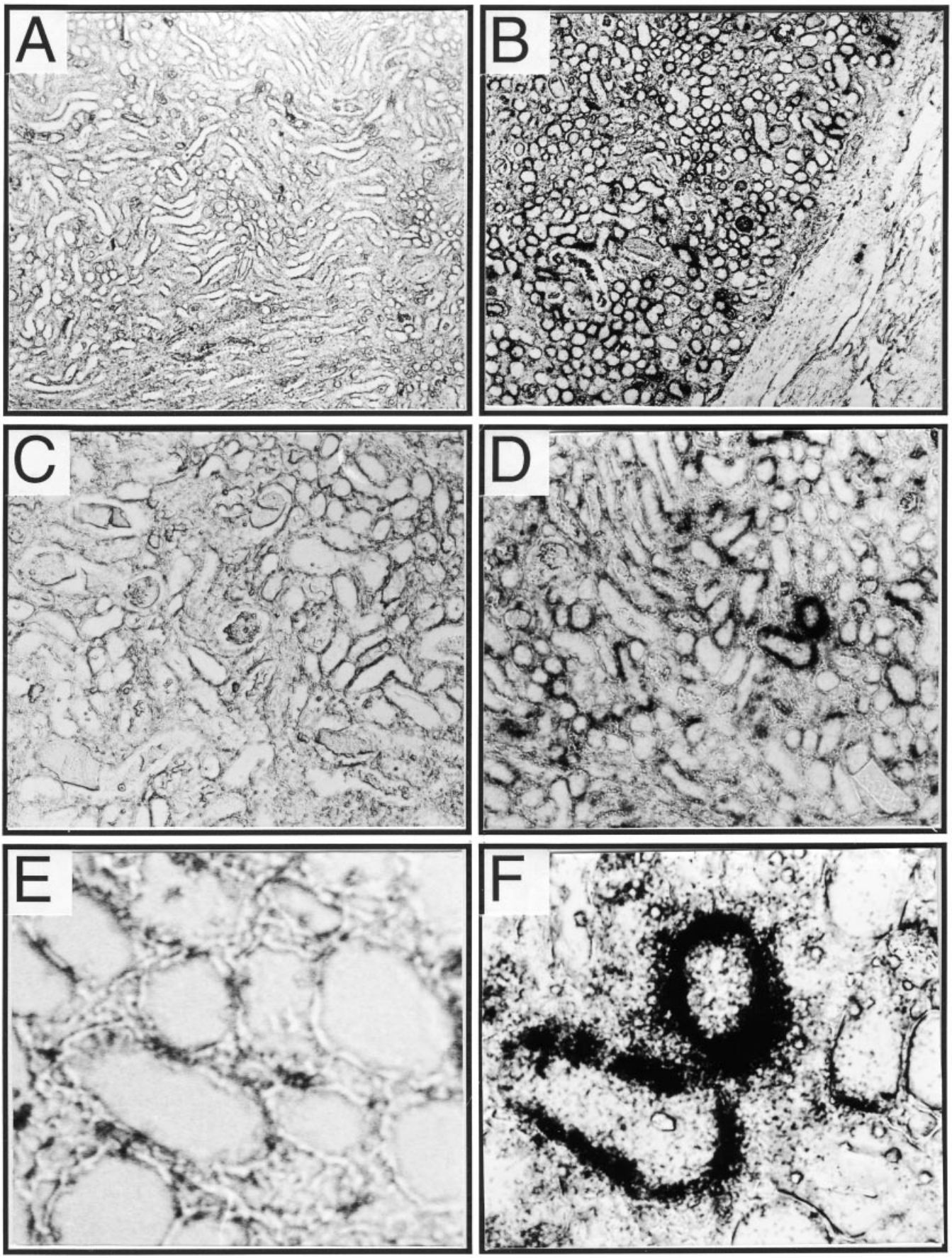
Lactoferrin, lysozyme, defensins, azurozidin, and different serine proteases, that is, N-elastase, cathepsin G, and proteinase 3, all contribute to the first line of defense against bacterial infections. Together they kill invading bacterias by, for example, depleting essential cofactors like free iron, degrading the bacterial cell wall components, or using other not yet fully elucidated mechanisms. In the kidney, various antibacterial substances have been identified in response to infections. However, it is not clear whether these proteins are present as a result of neutrophil infiltration or are actually synthesized by the resident kidney cells. The present findings, with the detection of LF protein and mRNA in the collecting tubules of the kidney, show that at least one of these antibacterial proteins can be produced by the kidney, which implies a role of the kidney in its own immune defense.

When comparing the immunohistochemical information with the pattern of mRNA expression, a clear difference was found. Only a few of the collecting tubules show apparent protein staining (Fig. 2 B, D), whereas the in situ hybridizations show massive expression in almost all of the tubules (Fig. 3 B, D, and F). These results suggest that LF is expressed and secreted throughout the collecting tubules and that LF receptors may be present only in the distal part of the tubules. In these latter regions, where LF may be reabsorbed, a massive accumulation of the protein is seen. A question arises about which receptor is responsible for such reabsorbtion. Studies in rats have previously shown that the excretion of iron is higher in fecal than in urine samples, further indicating the presence of a receptor-Fe-LF system for iron reabsorption in the kidney [28]. LF has previously been shown to bind to a number of cell surface proteins that were originally identified as receptors for other proteins. Two such receptors, LRP and gp330 (megalin), are expressed in rat kidney and have both been shown to bind LF in vitro [29]. These receptors may thus represent potential LF receptor candidates mediating endocytosis of iron bound to LF. However, gp330 is only present in proximal tubuli, arguing against a role in receptor-mediated endocytosis of LF produced by the kidney [30]. Furthermore, LRP is only expressed by dendritic interstitial cells and not by cells lining the distal tubules [31], indicating that it is not involved in LF reabsorption. Another not yet identified receptor(s) is therefore probably involved in this process. The presence of large amounts of LF protein in the tubuli but very low levels in urine also indicates additional functions of kidney LF. An interesting possibility is that LF participates in the iron metabolism by absorbing free iron from the urine, thereby making it available for metabolic use. It is also possible that as an iron chelator and antioxidant, renal tubular LF participates in antioxidant defense systems that protect the

Fig. 3. In situ hybridization of a human kidney sample (sample 6). (*A, C*, and *E*, negative control) Hybridization of a section from the medulla of a kidney with a LF sense probe (×4, ×10, and ×40). (*B*, *D* and *F*) Sections of human kidney incubated with a LF antisense probe.

Sense

Anti-sense



kidney against nonmicrobial oxidative injury, that is, ischemia-reperfusion injury and inflammation.

Substantial amounts of LF mRNA have also previously been detected in testis and prostate, indicating that LF may play an important role in the innate immunity also of these organs [7].

In summary, our results show that the kidney produces LF in a highly ordered fashion and that only a minor fraction of this LF is secreted into the urine. These findings indicate that LF has important functions in both the immune defense of the urinary tract and in the general iron metabolism. 1 It is also possible that LF has an important role in the antioxidant defense systems.

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